DECLARATION

I, Makoto AIHARA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Building, 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on November 11, 1998 under Patent Application No. 320102/1998 and that the following is a true and correct translation to the best of my knowledge and belief.

Dated: April 26, 2004

Makoto Aihara

Makoto AIHARA

[Title]	Application for Patent
[Reference Number]	98MCC058
[Filing Dat]	November 11, 1998
[Address]	To the Commissioner of the Patent Office
[International Classification]	C07K 19/00 C12N 9/00 C12N 15/09 C12N 15/52 G01N 33/00
[Title of the Invention]	A sandwich type fused macromolecule and a sensor using the same
[Number of Claims]	22
[Inventor]	
[Domicile or Residence]	c/o Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo
[Name]	Hiroshi YANAGAWA
[Inventor]	
[Domicile or Residence]	c/o Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo
[Name]	Nobuhide DOI
[Inventor]	
[Domicile or Residence]	c/o Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo
[Name]	Naoto NEMOTO
[Applicant for Patent]	
[Identification Number]	000005968
[Name]	Mitsubishi Chemical Corporation
[Administrator]	
[Identification Number]	100110607
[Patent Attorney]	
[Name]	Shinya MAYAMA
[Fee]	
[Number of register of payment]	062651
[Amount]	21000
[List f Attach d Documents]	
[Document's Name]	Specification 1
[Document's Name]	Drawing 1
[Document's Name]	Abstract 1
[Proof]	Required

[Name of Document] Specification

[Title of Invention] A sandwich type fused macromolecule and a sensor using the same

[Claims]

[Claim 1] A sandwich type fused macromolecule which comprises a first macromolecule portion having a signal conversion site and a second macromolecule portion having a molecule recognizing site which is inserted inside the first macromolecule portion.

[Claim 2] The macromolecule of claim 1 wherein the first macromolecule portion having a signal conversion site is natural or artificial protein.

[Claim 3] The macromolecule of claim 2 wherein the artificial protein is a mutant of a natural protein.

[Claim 4] The macromolecule of claim 2 wherein the artificial protein is a synthetic protein.

[Claim 5] The macromolecule of claim 1 wherein the first macromolecule portion having a signal conversion site is an enzyme protein having a catalytic activity, a fluorescent protein or a fluorescence-labeled protein.

[Claim 6] The protein of claim 5, wherein the enzyme protein is alkaline phosphatase, β-galactosidase, luciferase, glucose oxidase, or chloramphenicol acetyl transferase.

[Claim 7] The macromolecule of claim 5 wherein the fluorescent protein is Green Fluorescent Protein.

[Claim 8] The macromolecule of claim 1 wherein the first macromolecule portion having a signal conversion site is a natural nucleic acid or a mutant thereof which is selected from deoxyribonucleic acid, ribonucleic acid and peptide nucleic acid, or a nucleic acid derivative.

[Claim 9] The macromolecule of claim 8 wherein the natural

nucleic acid or a mutant thereof or the nucleic acid derivative is labeled with fluorescence.

[Claim 10] The macromolecule of claim 1 wherein the first macromolecule portion having a signal conversion site is a natural polysaccharide or a mutant thereof.

[Claim 11] The macromolecule of claim 10 wherein the polysaccharide or a mutant thereof is labeled with fluorescence.

[Claim 12] The macromolecule of claim 1 wherein the second macromolecule portion having a molecule recognizing site is a natural protein or a mutant thereof.

[Claim 13] The macromolecule of claim 1 wherein the second macromolecule portion having a molecule recognizing site is selected from enzyme proteins having a catalytic activity and proteins which can be bound to a low molecular substance or a high molecular substance.

[Claim 14] The macromolecule of claim 13 wherein the second macromolecule portion having a molecule recognizing site is selected from antibodies, proteins having a coenzyme binding ability, and ATP binding proteins.

[Claim 15] The macromolecule of claim 1 wherein the second macromolecule portion having a molecule recognizing site is a natural nucleic acid or a mutant thereof which is selected from deoxyribonucleic acid, ribonucleic acid and peptide nucleic acid, or a nucleic acid derivative.

[Claim 16] The macromolecule of claim 15 wherein the ribonucleic acid is selected from tRNA, rRNA mRNA and ribozyme.

[Claim 17] The macromolecule of claim 1 wherein the second macromolecule portion having a molecule recognizing site is a natural polysaccharide or a mutant thereof.

[Claim 18] A DNA which encodes the sandwich type fused

macromolecule of claim 1.

[Claim 19] A process for producing the sandwich type fused macromolecule of claim 1 wherein a host transformed with an expression vector containing a DNA encoding said sandwich type fused macromolecule is cultured, and said DNA is allowed to be expressed in said host.

[Claim 20] Use of the sandwich type fused macromolecule of claim 1 as a sensor.

[Claim 21] A method for screening a desired sandwich type fused macromolecule wherein a DNA encoding the sandwich type fused macromolecule of claim 1 is randomly mutated, and a desired macromolecule is screened from the mutated sandwich type fused macromolecules which are obtained by the expression of the randomly mutated DNA in a transformed host containing the above obtained randomly mutated DNA, based on the degree of the change of detection signal which is generated from the signal conversion site contained in the first macromolecule portion, which changes as a result of an interaction of a molecule recognizing site contained in the second macromolecule portion and a target molecule binding thereto.

[Claim 22] The method of claim 21 wherein the detection signal is a fluorescence.

[0001]

[Technical Field to which the Invention Belongs]

The present invention relates to a sandwich type fused macromolecule which comprises a first macromolecule portion having a signal conversion site and a second macromolecule portion having a molecule recognizing site which is inserted inside the first macromolecule portion, a DNA encoding said sandwich type fused macromolecule, a process for producing said sandwich type fused macromolecule, use of said sandwich type fused macromolecule as

a sensor, and a method for screening the sandwich type fused macromolecule as a desired sensor.

Biosensors are molecular sensors obtained by connecting a biological molecular recognition mechanism and a physical signal transduction technology. In order to construct a molecular sensor by using a molecular recognition ability of a biological macromolecule such as protein, it is necessary to link two allosteric sites (different sites), i.e., a molecular recognition site and a signal transduction site. The present invention relates to a general method for such linking and a molecule sensor obtained by this method. By the present invention, one can freely design a biosensor having an allosteric regulation function.

[0002]

[Prior Art]

There have been reported some examples of sensor proteins designed by means of protein engineering (Hellinga & Marvin 1998. Trends Biotechnol. 16, 183-189). For example, there have been known a cAMP sensor which uses the character that cAMP binds to a cAMP-dependent protein kinase to dissociate two subunits (Adams et al. 1991. Nature 349, 694-697); a maltose sensor using the character that when maltose binds to a maltose-binding protein, the structure of the maltose-binding protein changes (Marvin et al. 1997. Proc. Natl. Acad. Sci. USA 94, 4366-4371); a calcium ion sensor using the structural change of calmodulin (Miyawaki et al. 1997. Nature 388, 882-887; Romoser et al. 1997. J. Biol. Chem. 272, 13270-13274) and the like.

[0003]

[Object to be Solved by the Invention]

In the conventional sensor proteins, there have been used only the naturally occurring allosteric proteins where the subunits

dissociate or associate or the structures undergo a significant change when a target substance binds thereto. However, since the types of such macromolecules are limited, the types of molecules which these macromolecules can recognize are also limited. For example, when calmodulin or maltose-binding protein is used, only a sensor to calcium ion or maltose can be designed. However, there are a number of proteins that hardly change their structures by the binding of a target substance. For utilizing such various macromolecules, it has been a problem to link its molecule recognizing site with the signal conversion site and realize an allosteric regulation function.

[0004]

[Means for Solving the Object]

The present inventors have recently reported that it is possible to easily construct a sandwich type fusion protein wherein another protein is inserted into a protein (Doi et al. 1997. FEBS Lett. 402, 177-180). In conventional fusion proteins in which two proteins are coupled to each other at their ends, it is known that the activity of each of these two proteins is hardly affected by the fusion. In contrast, it is expected that, in the sandwich type fusion protein, the activity of one protein into which another protein is inserted is largely affected depending upon the difference in structural stability of the protein to be inserted. The present inventors have diligently studied. As a result, it has been found that a molecule sensor where detection signal is changed by the stabilization of the structure by the binding of a target substance with a molecule recognizing site, can be designed as required by inserting a macromolecule having a molecule recognizing site into a macromolecule having a signal conversion site, and thus the present invention has been completed.

[0005]

Namely, the present invention provides resides in

(1) A sandwich type fused macromolecule which comprises a first macromolecule portion having a signal conversion site and a second macromolecule portion having a molecule recognizing site which is inserted inside the first macromolecule portion.

(2) The macromolecule of (1) wherein the first macromolecule portion having a signal conversion site is natural or artificial protein.

(3) The macromolecule of (2) wherein the artificial protein is a mutant of a natural protein.

(4) The macromolecule of (2) wherein the artificial protein is a synthetic protein.

(5) The macromolecule of (1) wherein the first macromolecule portion having a signal conversion site is an enzyme protein having a catalytic activity, a fluorescent protein or a fluorescence-labeled protein.

(6) The protein of (5), wherein the enzyme protein is alkaline phosphatase, β-galactosidase, luciferase, glucose oxidase, or chloramphenicol acetyl transferase (CAT).

(7) The macromolecule of (5) wherein the fluorescent protein is Green Fluorescent Protein.

(8) The macromolecule of (1) wherein the first macromolecule portion having a signal conversion site is a natural nucleic acid or a mutant thereof which is selected from deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and peptide nucleic acid (PNA), or a nucleic acid derivative.

(9) The macromolecule of (8) wherein the natural nucleic acid or a mutant thereof or the nucleic acid derivative is labeled with fluorescence.

(10) The macromolecule of (1) wherein the first macromolecule portion having a signal conversion site is a natural polysaccharide or a mutant thereof.

(11) The macromolecule of (10) wherein the polysaccharide or a mutant thereof is labeled with fluorescence.

(12) The macromolecule of (1) wherein the second macromolecule portion having a molecule recognizing site is a natural protein or a mutant thereof.

(13) The macromolecule of (1) wherein the second macromolecule portion having a molecule recognizing site is selected from enzyme proteins having a catalytic activity and proteins which can be bound to a low molecular substance or a high molecular substance.

(14) The macromolecule of (13) wherein the second macromolecule portion having a molecule recognizing site is selected from antibodies, proteins having a coenzyme binding ability, and ATP binding proteins.

(15) The macromolecule of (1) wherein the second macromolecule portion having a molecule recognizing site is a natural nucleic acid or a mutant thereof which is selected from deoxyribonucleic acid(DNA), ribonucleic acid(RNA) and peptide nucleic acid(PNA), or a nucleic acid derivative.

(16) The macromolecule of (15) wherein the ribonucleic acid is selected from tRNA, rRNA mRNA and ribozyme.

(17) The macromolecule of (1) wherein the second macromolecule portion having a molecule recognizing site is a natural polysaccharide or a mutant thereof.

(18) A DNA which encodes the sandwich type fused macromolecule of (1).

(19) A process for producing the sandwich type fused macromolecule of (1) wherein a host transformed with an expression vector containing

a DNA encoding said sandwich type fused macromolecule is cultured, and said DNA is allowed to be expressed in said host.

(20) Use of the sandwich type fused macromolecule of (1) as a sensor.

(21) A method for screening a desired sandwich type fused macromolecule wherein a DNA encoding the sandwich type fused macromolecule of (1) is randomly mutated, and a desired macromolecule is screened from the mutated sandwich type fused macromolecules which are obtained by the expression of the randomly mutated DNA in a transformed host containing the above obtained randomly mutated DNA, based on the degree of the change of detection signal which is generated from the signal conversion site contained in the first macromolecule portion, which changes as a result of an interaction of a molecule recognizing site contained in the second macromolecule portion and a target molecule binding thereto.

(22) The method of (21) wherein the detection signal is a fluorescence.

[0006]

[Embodiment of the Practice of the Invention]

In the sandwich type fused macromolecule of the present invention, a second macromolecule portion having a molecule recognizing site for recognizing the target molecule is inserted into a first macromolecule portion having a signal conversion site. The first macromolecule portion having a signal conversion site is typically a natural or artificial protein. The artificial protein includes a mutant of a natural protein and a synthetic macromolecule obtained by organic synthesis.

Specifically, the first macromolecule portion having a signal conversion site is an enzyme protein having a catalytic activity, a fluorescent protein, a protein labeled with a fluorescent substance, or the like. More specifically, the examples of the enzyme protein

include alkaline phosphatase, β-galactosidase, luciferase, glucose oxidase, or chloramphenicol acetyl transferase (CAT). In addition, a macromolecule which can generate a signal detectable by a physical means, such as Green Fluorescent Protein (GFP) having a fluorescent chromophore is also included in the examples of the above first macromolecule. Further, the examples of the first macromolecule portion having a signal conversion site include a natural nucleic acid or a mutant thereof such as DNA(deoxyribonucleic acid), RNA(ribonucleic acid) and PNA(peptide nucleic acid), or a nucleic acid derivative, or those labeled with fluorescence; a natural polysaccharide or a mutant thereof, or those labeled with fluorescence. To be short, the first macromolecule portion having a macromolecule signal conversion site may be any one so long the activity of signal conversion site can be greatly changed by the difference of structural stability of the second macromolecule portion which is generated by interaction between the molecule recognizing site of the second macromolecule portion and its target molecule.

[0007]

The second macromolecule portion having a molecule recognizing site is a natural protein or a mutant thereof, and examples thereof include enzyme proteins having a catalytic activity and proteins which can be bound to a low molecular substance or a high molecular substance. Examples of such second macromolecule portion include antibodies, proteins having a coenzyme binding ability, and ATP binding proteins. In addition, the examples of the second macromolecule portion include a natural nucleic acid or a mutant thereof which is selected from DNA, RNA (tRNA, rRNA, mRNA, ribozyme, and the like) and PNA, or a nucleic acid derivative; a natural polysaccharide or a mutant thereof. To be short, the second

macromolecule portion having a molecule recognizing site is a macromolecule which can selectively recognize a target molecule and can bind or interact therewith, and it is typically an enzyme which specifically recognizes a substrate.

[0008]

The sandwich type fused macromolecule of the present invention which has a sequence wherein a sequence of the second macromolecule portion having a molecule recognizing site is inserted inside the first macromolecule portion having a signal conversion site, can be synthesized by any general technique for synthesis of macromolecules, such as genetic engineering, chemical synthesis or enzymatic synthesis. When the sandwich type fused macromolecule of the present invention is a macromolecule having a peptide sequence, it is advantageous to synthesize it using a recombinant DNA technique. For example, based on the amino acid sequence of the sandwich type fused macromolecule of the present invention, DNA encoding it is synthesized by conventional method, and the DNA is inserted into an expression vector containing a promoter, a terminator and the like in such a manner that the DNA can be expressed. A suitable host cell is transformed with the thus-obtained vector and the transformed cell is cultured, whereby the sandwich type fused macromolecule of the present invention can be prepared. In place of the aforementioned synthetic DNA, a naturally occurring DNA encoding the first macromolecule portion is cut with a suitable restriction enzyme, and then a naturally occurring DNA encoding the second macromolecule portion, which has two ends which can be linked with the two DNA fragments obtained by the cutting, is linked with the two cut ends of the DNA encoding the cut first macromolecule portion, whereby the DNA encoding the second macromolecule portion can be inserted into the naturally occurring DNA encoding the first

macromolecule portion. Further, the desired two DNA fragments encoding the N-terminus side and C-terminus side of the first macromolecule portion can be synthesized from the naturally occurring gene encoding the first macromolecule portion by using suitable plural primers. Two fragments of the DNA encoding the first macromolecule portion of the sandwich type fused macromolecule of the present invention can be synthesized by inserting a proper restriction enzyme site into the naturally occurring DNA encoding the first macromolecule portion by a PCR method using a primer having a proper sequence and cutting the resultant DNA in the above manner. The thus-obtained two fragments of the DNA encoding the first macromolecule portion and the DNA fragment encoding the second macromolecule portion are linked in accordance with a usual method using a ligase, whereby a DNA fragment encoding the sandwich type fused macromolecule of the present invention, which has a desired sequence, can be obtained.

[0009]

The thus obtained DNA sequence which encodes the sandwich type fused macromolecule is operatively linked to a suitable expression control sequence (promoter). Examples of the promoter includes phage λ PL promoter、T7 promoter; lac, trp, lpp and tac promoters of E. coli; SPO1 promoter, penP promoter of bacillus bacterial; pho5 promoter, PGK promoter, GAP promoter, ADH1 promoter, SUC2 promoter, GAL4 promoter, Mf α promoter of yeasts; polyhedron promoter, P10 promoter of insect cell; SV40 early and late promoters for animal cells; retrovirus LTR promoter, CMV promoter, HSV-TK promoter, metallothionein promoter; 35S promoter, a promoter of rice actin gene for plant cells; and the like. The expression vector containing the DNA sequence encoding the sandwich type fused macromolecule of the present invention contains a DNA region to

be transcribed, and signal sequences for starting and terminating transcription. Generally, the expression vector contains an expression control regions which works with a repressor binding site and an enhancer. In addition, the expression vector contains a selection marker. The suitable marker includes a dihydrofolic acid reductase (dhfr) gene, a neomycin resistance gene for eukaryotic cells, and a tetracycline or ampicillin resistance gene for bacteria. The dhfr gene confers methotrexate resistance to the transformed cell, and the neomycin resistance gene confers G418 resistance to the transformed cell. When a host is a dhfr gene-defective CHO cell and the dhfr gene is used as the selection marker, it is possible to select the transformant in a thymidine-free culture medium. In this case, cultivation is carried out while gradually increasing methotrexate (MTX) concentration, to select resistant strains, thereby the DNA sequence encoding the sandwich type fused macromolecule of the present invention is amplified in the resistant cell together with the dhfr gene, and a CHO (dhfr-) cell of high level expression can be obtained.

[0010]

If necessary, the above-mentioned expression vector may be constructed in such a way that a signal sequence is added to the N-terminus of the protein. Such a signal sequence is PhoA signal sequence, SUC2 signal sequence or the like for E. coli host, and it is Mfα signal sequence, SUC2 signal sequence or the like for yeast host, and it is α-interferon signal sequence or the like for animal cell host.

[0011]

The host cell in which the DNA sequence encoding the sandwich type fused macromolecule of the present invention is expressed includes eukaryotic cells such as mammalian cells, plant cells,

insect cells, yeast and aspergillus; and prokaryotic cells such as bacterial cells. The above expression vector is introduced into a host cell by calcium phosphate transfection, electroporation, transduction, infection or other method.

The sandwich type fused macromolecule of the present invention can be expressed in the above-mentioned host such as mammalian cells, yeasts or bacteria under the control of the above promoter. The prokaryotic host includes, for example, E. coli, Bacillus subtilis, Salmonella, Pseudomonas, Streptomyces, Staphylococcus and the like. Yeasts ncludes, for example, Saccharomyces cerevisaie, Schizosaccharomyces pombe, Pichia pastoris and the like. Mammalian cells includes, for example, COS-7 cell, mouse AtT-20 cell, rat GH3 cell, rat MtT cell, mouse MIN6 cell, Vero cell, C127 cell, CHO cell, dhfr gene-defective CHO cell, HeLa cell, L cell, BHK cell, BALB 3T3 cell, 293 cell, Bows melanocyte and the like.

[0012]

The transformed prokaryotic host is grown and cultured in a liquid medium containing a carbon source (e.g., glucose, dextran, soluble starch, etc.), a nitrogen source (e.g., an ammonium salt, a nitric acid salt, peptone, casein, meat extract, bean cake, etc.) and an inorganic substance (e.g., calcium chloride, sodium dihydrogen phosphate, magnesium chloride, etc.) at an appropriate pH (pH of about 5 to 8) for an appropriate period of time (about 3 to 24 hours). When an expression vector containing an inducible promoter is employed, induction is performed with temperature or a chemical inducing substance. The appropriate cultivation temperature is in a range of about 14 to 43℃ in the case of E. coli or in a range of about 30 to 40℃ in the case of Bacillus bacteria. After the culturing, the cell is disrupted by a physical or chemical method, and the sandwich type fused macromolecule of the present

invention is purified from the resultant crude extract. While the sandwich type fused macromolecule obtained in the above manner can be purified by a usual method, it is advantageous to produce a sandwich type fused macromolecule in which a plurality of histidine residues are added to the N-terminus and to purify it by an affinity chromatography.

Alternatively, the cell is cultured by a known method when the host is yeast, a mammalian cell or an insect cell, and the produced sandwich type fused macromolecule of the present invention may be purified by a known method.

[0013]

When the sandwich type fused macromolecule obtained by the above method functions as a sensor, the following procedure is not necessary. However, when a further improvement of the function as a sensor is desired, a procedure for introducing a mutant into the sequence of the macromolecule for improvement is necessary. In this case, (1) a site-specific mutation is introduced and a sequence is designed, or (2) a library wherein random mutations are introduced is prepared, and a sensor macromolecule which greatly change the signal only when a target molecule is bound therewith is screened from the library. Specifically, error-prone PCR is carried out by using a plasmid containing DNA encoding the sandwich type fused macromolecule of the present invention as a template and adding manganese ion to amplify DNA fragment encoding the central region and the C-terminal region of the first macromolecule portion, and thus random point mutation is introduced into a region before and after the second macromolecule portion. From the mutated sandwich type fused macromolecule which is obtained by the expression of the obtained random mutated DNA, a desired sandwich type fused macromolecule can be screened based on the degree of the change

of detection signal which is generated from the signal conversion site contained in the first macromolecule portion, which changes as a result of an interaction of a molecule recognizing site contained in the second macromolecule portion and a target molecule binding thereto. The aforementioned recombinant DNA techniques for producing the sandwich type fused macromolecule of the present invention are described in many recombinant experiment manuals, and can be easily carried out, and modified methods of the above may be used.

[0014]

Examples of the light sensor protein of the present invention include a light sensor protein which is obtained by inserting any second macromolecule portion having a molecule recognizing site inside the Green Fluorescent Protein which is a first macromolecule portion having a signal conversion site, and which generates an increased fluorescence by the stabilization of the structure by binding of a target molecule (see Fig. 1). As shown in Fig.1, any molecule having a molecule recognizing site is inserted inside another macromolecule having a signal conversion site, and if its structure is stabilized by binding of a target molecule, detection signal is changed.

[0015]

The sandwich type fused macromolecule of the present invention is a molecular sensor which incorporates specific molecule recognizing mechanisms of various macromolecules, which mechanism has not been usable heretofore. By the present invention, it becomes possible to design and screen such a molecular sensor as required. By using the sensor protein of the present invention, it becomes possible to detect a particular molecule (cell, protein, virus, antibody, DNA, electrolyte, drug, insecticide, environment hormone,

other low molecular compound and the like) contained in a complex solution. The sensor protein is widely applied and can be utilized in various fields such as cell biology, biomedicine, environment, bio-computer, and other fields.

[0016]

[Examples]

The present invention will be described more in detail below with reference to Examples. However, Examples described below are provided for recognizing specific embodiments of the present invention, and the scope of the present invention is by no means restricted by the Examples below.

[0017]

Example 1

Preparation of GFP sensor which recognizes β-lactamase inhibitor

β-lactamase was selected as a macromolecule having a molecule recognizing site. The X-ray crystallographic structure of sole β-lactamase as well as that of the complex of β-lactamase and β-Lactamase Inhibitory Protein (BLIP) have been determined. It is known that the structure of β-lactamase is hardly changed by binding of BLIP (Strynadka et al. 1996. Nature Struct. Biol. 3, 290-297). On the other hand, as the first macromolecule portion having a signal conversion site, green fluorescence protein (GFP) was selected. GFP has a fluorescent chromophore in its insides and generates green fluorescence by irradiation of ultraviolet light. First, a fusion protein was constructed by inserting β-lactamase into the loop site between 172 Gln and 173 Asp of GFP. Further, a sensor protein wherein the fluorescent intensity of GFP was increased by binding of BLIP was screened from the library obtained by introducing a random mutation into the protein obtained above.

[0018]

Material: E. coli JM109, and plasmids pUC18 and pUC4KIXX (Barany 1985. Gene 37, 111-123) and pGFPuv (Crameri et al. 1996. Nature Biotechnol. 14, 315-319) were provided by Dr. Itaya of Mitsubishi Chemical Corporation, Institute of Life Science. Plasmid pEOR (Prijambada et al. 1996. FEBS Lett. 382, 21-25) was provided by Dr. Yomo of Osaka University and BLIP gene (Doran et al. 1990. J. Bacteriol. 172, 4909-4918) was provided by Dr. Schroeder of Alberta University (Canada). The following commercially available enzymes and reagents were used respectively: Restriction enzymes AflIII, BspHI, KpnI, NcoI, NdeI, NheI and XbaI (NEB), Eco47III and EcoRI (Toyobo Co., Ltd.), HindIII and SacI (Boehringer Mannheim), and XhoI (Takara Shuzo Co., Ltd.); gene kits Ligation High and Blunting High (Toyobo Co., Ltd.); heat-resistance enzymes Taq DNA Polymerase (Grainer) and Vent DNA Polymerase (NEB); and carbenicillin (Sigma), and kanamycin, L-arabinose and D-glucose (Wako Pure Chemical Industries., Ltd.), IPTG (Nacali Tesque, Inc.); Ni-NTA resin (QIAGEN). The basic operations of gene engineering (e.g., cloning, transformation and cultivation of E. coli, collection of plasmids and the like) were carried out according to the experimental manual of Molecular Cloning, A Laboratory Manual, 2nd Ed., (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York(1989)).

[0019]

Construction of Plasmid

The plasmid pND101 (Fig. 2) was constructed in accordance with the following procedure. First, BAD promoter and AraC protein gene that controls the expression of a gene under BAD promoter (Guzman et al. 1995. J. Bacteriol. 177, 4121-4130) were amplified by PCR using the genome of E. coli JM109 strain as the template and primers (SEQ ID NOS: 1 and 2), digested with NcoI and NdeI and cloned in AflIII - NdeI site of pEOR. Then, the resultant plasmid was digested

with BspHI to delete ampicillin-resistant gene which was substituted with kanamycin-resistant gene contained in the AflIII - XhoI fragment of pUC4KIXX. Further, PCR fragments obtained by amplifying BLIP gene with primers (SEQ ID NOS: 3 and 4) were digested with NdeI and SacI, and inserted into the downstream of BAD promoter. By this procedure, the expression of BLIP can be induced in the presence of L-arabinose and suppressed in the presence of D-glucose. The resultant plasmid was digested with SacI and HindIII to delete its HindIII site, and treated to make blunt ends, and then it was self-cyclized to obtain a plasmid pBAD-BLIP-Kn. On the other hand, in order to introduce a restriction enzyme site for inserting β -lactamase gene into GFP gene, DNA fragments of the N- and C-terminus of GFP were amplified by PCR using pGFPuv as a template and two sets of primers (SEQ ID NOS: 5 and 6, and SEQ ID NOS: 7 and 8), and each of the DNA fragments was digested with NheI - KpnI or KpnI - SacI respectively, and the DNA fragment thus digested were inserted at once into the NheI - SacI site of pEOR. As the result, a HindIII - KpnI - EcoRI site was inserted between the 172 Gln and 173 Asp of GFP and at the same time, a sequence encoding 6 histidine residues was added at the N-terminus of GFP. The thus-obtained mutant GFP gene was amplified by PCR using primers (SEQ ID NOS: 8 and 9) and inserted into Eco47III site of the pBAD-BLIP-Kn. At this time, in order to bring an expression amount of the mutant GFP to the same level as that of BLIP located in the downstream of BAD promoter, the 6 bases corresponding to -35 region of tac promoter contained in the primer (SEQ ID NO:9) were made to be random, and a pND101 was finally obtained by selecting one which contained a promoter having the same strength level as BAD promoter.

[0020]

Insertion of β-lactamase gene and screening

The β-lactamase gene on the pUC18 was amplified with primers (SEQ ID NOS: 10 and 11), digested with HindIII - EcoRI and inserted into the HindIII - EcoRI site of GFP gene on the pND101 (Fig.3). Each of these primers contains a random sequence consisting of 9 bases as a linker between β-lactamase gene and GFP gene. This DNA library was transferred to E. coli JM109, and the E. coli was cultured on a LB plate containing 10 μg/ml of kanamycin to allow the E. coli to form a colony. 100 colonies were arbitrarily selected and each of the colonies was plated on two plates, each of which contains 0.2% L-arabinose or 0.2% D-glucose respectively, to allow the E. coli to again form a colony, and a colony wherein the intensity of fluorescent generated under ultraviolet light is different between the two plates, was selected. The selected colony was cultured in a liquid medium, plasmids were collected and Error-prone PCR (Buchholz et al. 1998. Nature Biotechnol. 16, 657 - 662) was carried out by using the thus collected plasmids as a template and primers (SEQ ID NOS: 12 and 8) to introduce a random mutation into the region containing β-lactamase. The resultant PCR fragment was digested with XhoI and XbaI and inserted into the same site of the original plasmid to form a library of plasmids, and clones which indicated a larger difference in the intensity of fluorescence was selected from the library. These steps of random mutation introduction and selection were repeated two times. The finally obtained E. coli containing a sensor protein indicated weak fluorescence when no BLIP was not coexpressed (i.e. on the plate containing glucose) but indicated strong fluorescence when BLIP was coexpressed (i.e. on the plate containing arabinose). Namely, a sensor protein that showed an increase of the fluorescent intensity by binding with BLIP in the cell was obtained.

[0021]

Purification of a sensor protein and BLIP

For confirming that the selected protein actually acts as a sensor by itself in vitro, the sensor protein and BLIP were purified. E. coli JM109 having the plasmid encoding the sensor protein was cultured in a 2xYT medium containing 20 μg/ml of kanamycin at 37℃ until OD_{600} became 0.6, and then, IPTG was added thereto in a final concentration of 1 mM and cultivation was continued further for 5 hours. The sensor protein expressed in large scale was accumulated mainly in an insoluble fraction. The collected E. coli was disrupted by sonication and the insoluble fraction was collected by centrifugation. The insoluble fraction was dissolved with 8M urea, and the sensor protein having a histidine tag at its N-terminus was loaded on a Ni-NTA column, washed, and eluted by decreasing pH. Urea was removed from the fraction containing the protein of interest by dialysis to renature the sensor protein. On the other hand, BLIP gene was amplified by PCR using primers (SEQ ID NOS: 13 and 4) for adding 6 histidine residues to the N-terminus, digested with NdeI and SacI and inserted into the same site of the pEOR. E. coli JM109 having this plasmid was cultured in a 2xYT medium containing 100 μg/ml of carbenicillin, and BLIP was expressed in a large scale and purified in the same way.

[0022]

Measurement of fluorescence of sensor protein

The fluorescence spectra of the sensor protein in the presence and absence of BLIP were respectively measured with Shimazu RF502 spectrofluorometer. The excitation spectrum at a radiation wave length of 505 nm of the sensor protein obtained by inserting β-lactamase into GFP indicated two absorption maxima around 395 nm and around 475 nm like wild type GFP (Fig. 4). When BLIP was added to this sensor protein, the intensity of fluorescence spectrum

increased almost two times. However, when BSA was added as a control, no change of intensity was observed. Thus, it has been showed that this protein works as a sensor for specifically recognizing BLIP.

[0023]

[Effect of the Invention]

By using the sandwich type fused macromolecule of the present invention as, for example, a GFP sensor which can recognize certain molecule, fluorescent imaging of various molecules in living cells can be carried out. The molecule recognizing GFP sensor can bind with a molecule and generate fluorescence by only expressing the gene in cells, and thus gene transfection is available in any cells. Also, the molecule recognizing GFP sensor can be used as a bio-computer element. While the bacteriorhodopsin which is currently expected as a bio-computer element is an element which recognizes light and converts it into molecular information, the molecule recognizing GFP sensor can be expected to play a complementary role as an element which recognizes certain molecule and converts it into light.

[0024]

SEQUENCE LISTING

<110> Mitsubishi Chemical Corporation

<120> Sandwich type fusion high molecule and a sensor using the same

<130> 001

<140>

<141> 1998 - 11-

<160> 13

<170> PatentIn Ver. 2.0

<210> 1

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 1

gcgccatggt tgcataatgt gcctgtcaaa tggac 35

<210> 2

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 2

cgccatatgt tcactccatc caaaaaaacg ggtatg 36

<210> 3

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 3

gcccatatgg cgggggtgat gaccg 25

<210> 4

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 4

gccgagctct tatacaaggt cccactgccg 30

<210> 5

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 5

gccgctagcc atcatcatca tcatcatggt atgagtaaag gagaagaact tttc 54

<210> 6

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 6

gccggtaccc caagcttttc aatgttgtgg cgaattttg 39

<210> 7

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 7

gccggtacca gaattcgatg gaagcgttca actag 35

<210> 8

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 8

gccgagctct ctagattatt tgtatagttc atccatgcc 39

<210> 9

<211> 44

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 9

gcatcccggg cnnnnnnttt aatcatcggc tcgtataatg tgtg 44

<210> 10

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 10

gccaagcttn nbnnbnnbca cccagaaacg ctggtg 36

<210> 11

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 11

gccgaattcv nnvnnvnncc aatgcttaat cagtgaggc 39

<210> 12

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 12

aaactcgagt acaactataa ctc 23

<210> 13

<211> 43

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic DNA

<400> 13

gcccatatgc atcatcatca tcatcatgcg ggggtgatga ccg 43

[Free text of the Sequence Listing]

SEQ ID NOS:1-13: Synthetic DNA

[Brief Description of the Drawings]

[Fig.1]

A conceptional diagram of the molecule recognizing sensor. Any macromolecule having a molecule recognizing site is inserted inside another macromolecule having a signal conversion site. When the structure is stabilized by binding of a target molecule, detection signal is changed.

[Fig.2]

A diagram showing the gene map of vector pND101 for screening and large scale expression. Expression of BLIP gene contained in the above vector is induced in the presence of L-arabinose and suppressed in the presence of D-glucose, by a regulator AraC of BAD promoter. GFP gene contains a restriction enzyme site where β-lactamase gene is to be inserted.

[Fig.3]

A diagram showing construction of the sequence of gene DNA which shows the insertion of β-lactamase gene into GFP gene. Each linker positioned at both ends of β-lactamase contains a restriction

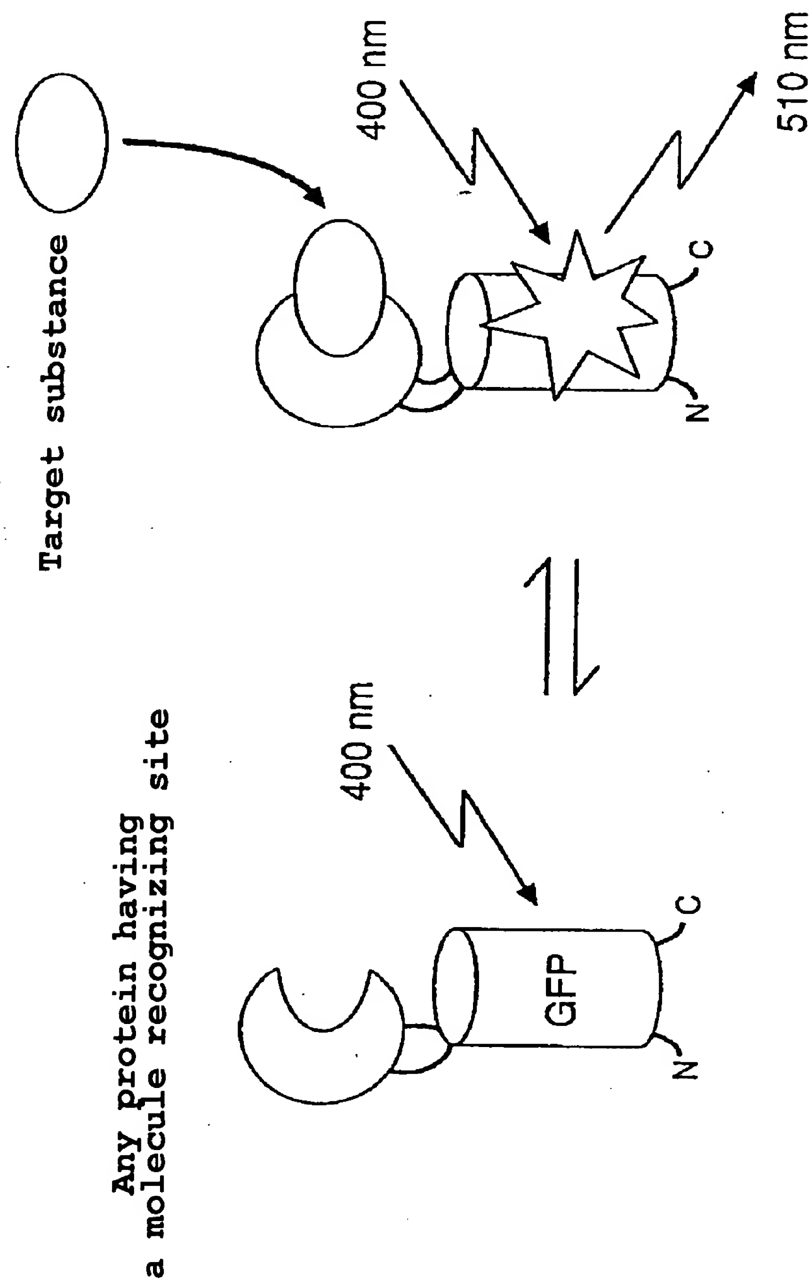
enzyme site of 6 bases and a random sequence of 9 bases.

[Fig.4]

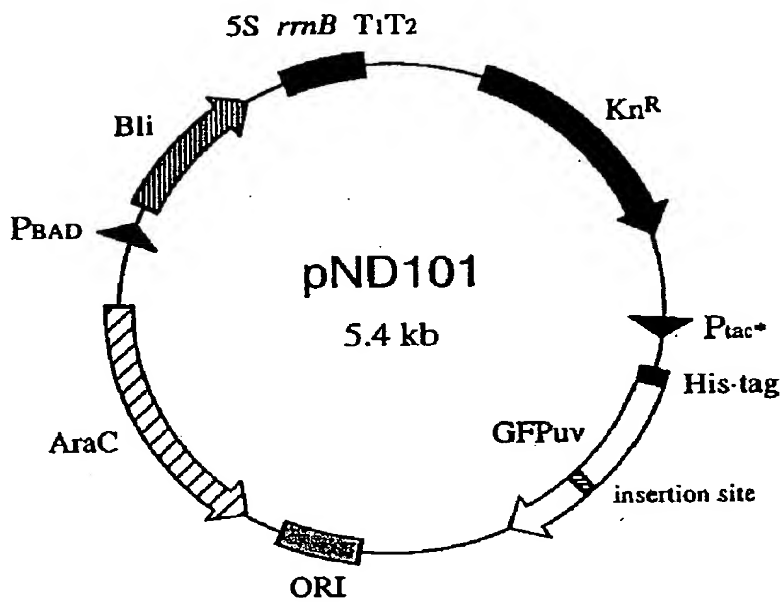
(a) fluorescent spectrum for the sensor protein (a concentration of 0.3 μM), (b) fluorescent spectrum when 7 μM of BLIP was added and (c) fluorescent spectrum when 7 μM of BSA (bovine serum albumin) was added.

[Name of Document] Drawing

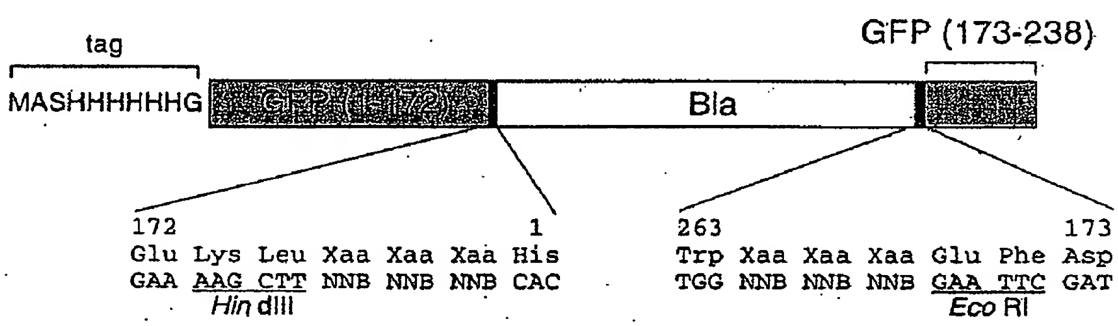
[Fig.1]



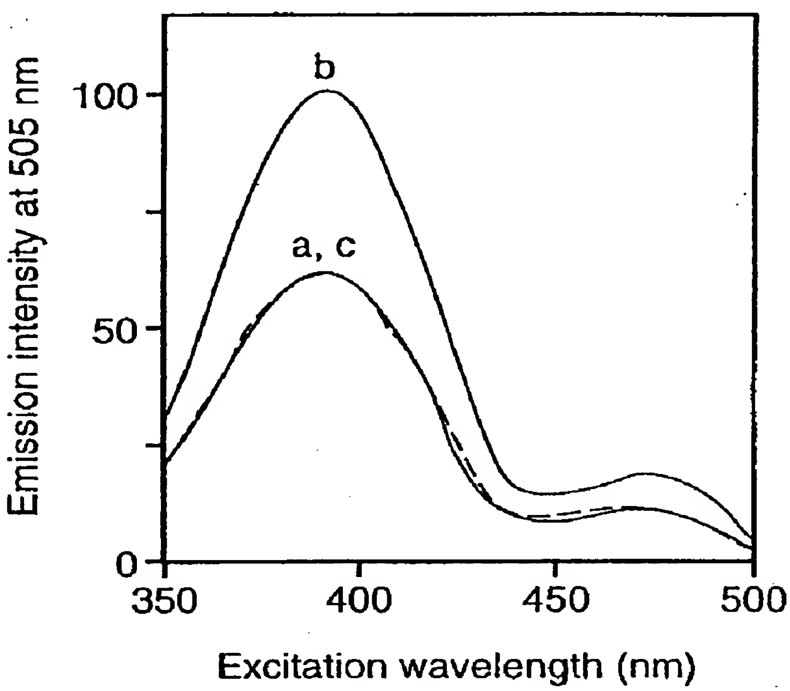
[Fig.2]


5S rrnB T1T2
KnR
Bli
PBAD
pND101
5.4 kb
Ptac*
His-tag
GFPuv
AraC
insertion site
ORI

[Fig.3]


tag
MASHHHHHHG
GFP (1-172)
Bla
GFP (173-238)
172
Glu Lys Leu Xaa Xaa Xaa His
GAA AAG CTT NNB NNB NNB CAC
Hin dIII
1
263
Trp Xaa Xaa Xaa Glu Phe Asp
TGG NNB NNB NNB GAA TTC GAT
Eco RI
173

[Fig.4]


b
a, c
100
50
0
350
400
450
500
Emission intensity at 505 nm
Excitation wavelength (nm)

[Name of Document] Abstract

[Abstract]

[Object] To provide a sandwich type fused macromolecule which is obtained by linking a biological molecule recognizing mechanism and a physical signal conversion technique, and has an allosteric regulation function, and is useful as a biosensor.

[Means for Solution] A sandwich type fused macromolecule which comprises a first macromolecule portion having a signal conversion site and a second macromolecule portion having a molecule recognizing site which is inserted inside the first macromolecule portion, a DNA which encodes the sandwich type fused macromolecule, a process for producing the sandwich type fused macromolecule, use of the sandwich type fused macromolecule as a sensor, and a method for screening the sandwich type fused macromolecule as a desired sensor, are provided.

[Effect] The sandwich type fused macromolecule of the present invention is a molecule sensor wherein various specific molecule recognizing mechanisms of macromolecules, which have not ever utilized, are incorporated. By freely design the biosensor, screening of active molecules can be carried out. The sensor of the present invention can be utilized in many fields such as cell biology, biomedicine, environment and bio-computer.

[Selected Drawing] Fig.1